Chapter 7
Purification and Characterization of Dendrimers

Various methods of dendrimers characterization have been deeply reviewed such as NMR, electron paramagnetic resonance (EPR), mass spectrometry, UV–VIS spectrometry, time-resolved and nonlinear optical spectroscopy, laser light scattering, optical rotation, circular dichroism (CD), synchrotron radiation-based circular dichroism (SRCD), IR, Raman, fluorescence, X-ray diffraction, small-angle X-ray scattering (SAXS), small-angle neutron scattering, atomic force microscopy (AFM), scanning tunneling microscopy (STM), optical tweezers, transmission electron microscopy (TEM), isothermal titration calorimetry (ITC), different chromatographic and electromigration methods (electrophoresis, capillary electrophoresis), dielectric spectroscopy, differential scanning calorimetry, etc. [7, 10, 13, 23, 24, 27, 30, 38, 42, 52, 53, 57]. Hence, this chapter is focused only on some ways, which are important for dendrimer characterization. Their con and pros are clearly discussed.

Dendrimers can be characterized and purified by separation methods, as well as they can be employed as stationary phase and/or selection factors improving the efficacy of separation techniques [30, 50]. The two main classes of separation techniques are chromatographic and electromigration methods. Moreover, it is mandatory to use methods differing in the separation principle in order to check preparative efficiency by other complementary separation techniques, such as RP-HPLC and CE [10, 29, 42, 50, 57, 69]. Unfortunately, many researchers check the final purity after separation using the same technique. This violated the main principle of complementary characterization of separated product. Moreover, even combination of two complementary techniques is not always sufficient for removal of all by-products differing by only a single modification or deletion (the negligible difference such as missing one amino acid in molecules with mass above 7 kDa).

J. Šebestík et al., *Biomedical Applications of Peptide-, Glyco- and Glycopeptide Dendrimers, and Analogous Dendrimeric Structures*,
DOI 10.1007/978-3-7091-1206-9_7, © Springer-Verlag Wien 2012

7.1 Chromatographic Methods

Dendrimers were applied for improvement of stationary phase selector. Multivalency as one of main factors influencing the chiral separation HPLC was studied using enantioselective polymeric support with aliphatic dendrons [37]. The bigger distance of the selector from the support core, the more active it is. These materials provided effective separation of enantiomers. The symmetry of selector played crucial role for specific selectivity of the chiral stationary phase. Perfect dendrons prepared by convergent approach in solution were much better chiral selectors than those obtained by solid-phase divergent method.

In contrast to the chiral selector, dendronized stationary phases based on melamine had lower sequestration capacity for perfectly made dendrons prepared by convergent methods [1]. The sequestration capacity is increasing proportionally to the generation of dendrons.

Another example of stationary phase modification is usage of PAMAM-dendronized silica for size-exclusion chromatography [47].

Surface transformation of PAMAM dendrimers and the product quality can be easily monitored by ultra-performance liquid chromatography (UPLC) [11]. As expected, UPLC provides a vastly improved analytical method for the characterization of dendrimer polydispersity and variance in a typical surface modification in comparison with HPLC. In the presented example, the surface modification of G4 PAMAM-$(NH_2)_{64}$ dendrimer with biotin was studied. UPLC with increased average number of theoretical plates by a factor of 7 and reduced retention times of analytes by 36 % enhanced the resolution capability for discrimination of surface variances in dendrimers. Moreover, the reduced band spreading during the separation process maintained higher concentration of the analytes at the point of detection. This led to decrease of injection volumes and improvement of detection limit by a factor of 100, i.e., UPLC detection limit for G4, PAMAMs, was 1.69×10^{-12} mol. Thus, the UPLC outperforms HPLC in the detection, purification, and separation of unmodified as well as surface-modified PAMAM dendrimers.

Also, with certain limitations, the simple RP-HPLC method with UV detection can serve for quantification of peptide dendrimers. The technique was applied for assessment of skin permeation experiments [41].

Preparative HPLC can be also a shortcoming of MAP synthesis by conjugation approach [33]. The "expensively" purified peptide is conjugated with high excess (12-fold for tetravalent MAP) in order to achieve completion of linking. Hence, the direct synthesis with one HPLC purification of final product could be more competitive in MAP assembly than a convergent synthesis with several HPLC purifications.

For further comparison of HPLC and UPLC (robustness and ruggedness) see [16, 65].

Rapid and useful reaction monitoring by high-performance thin-layer chromatography (HPTLC) was described. The spot of separated compound can be characterized by MALDI-TOF-MS [8].

New fullerene glycodendron conjugates were prepared by the Diels–Alder reaction between C_{60} and anthryl glycodendron containing D- or L-gluconamides at the ends [62]. Moreover, two optically pure diastereomeric fullerodendrons were isolated from the mixture of diastereomers by silica gel column chromatography and GPC and characterized by ^{1}H and ^{13}C NMR spectroscopy, FTIR, and MALDI-TOF-MS analysis.

Average molecular weights of hyperbranched 5,6-glucan obtained by multi-angle laser light scattering (MALLS) were in range 7,400–122,400, in contrast to significantly smaller average molecular weight obtained by size-exclusion chromatography (SEC) [63]. These results when combined with viscosity measurements suggested an existence of these polymers in a compact spherical conformation.

Size-exclusion chromatography was found useful for purification of two glycocluster ligands with cyclam core bearing thiourea-linked glucose and 2-acetamido-2-deoxy-glucose at the periphery [61]. According to isothermal titration microcalorimetry, these glycoclusters interacted with Con A. Their stable complexes with the diagnostically relevant radioisotopes ^{99m}Tc and ^{64}Cu can be formed.

Gel permeation chromatography of sulfated oligosaccharide cluster with lysines revealed a narrow distribution with polydispersity index 1.08 [25]. The polylysine-dendritic sulfated cellobiose was analyzed by NMR and FTIR spectroscopies.

7.2 Electromigration Methods

Since capillary electrophoresis, capillary electrochromatography, and other electromigration methods are complementary to chromatographic methods [7, 10, 12, 29, 43, 46, 53, 54, 57, 58, 69], they can serve as independent purity assessment after purification of peptides, glycopeptides, and glycopeptide dendrimers by HPLC and UPLC.

Dendrimers play a dual role in electromigration methods, i.e., they are both the subject and object of separation [50]. For instance, dendrimers can serve as pseudo-stationary phase (PSP) for separation of other dendrimers [43]. When dendrimers served as modifier of the background electrolyte, higher performance of separation is achieved, in contrast to classical micellar electrokinetic chromatography [46]. The effect was explained by a higher homogeneity of the dendrimer phase and a broader migration time window [46]. Obviously, the separation was influenced by the size and charge of the dendrimers as well as by the composition of the electrolyte. The resolution is proportional to the concentration of dendrimer [43].

Both polyacrylamide gel (PAGE) and capillary electrophoreses (CE) were used for investigation of PAMAM dendrimers and their derivatives [9]. CE achieved separation up to the fifth generation of ammonia-core PAMAM dendrimers.

If the separation of negatively charged PAMAM succinamic acid dendrimers (PAMAM-SAH) (G1–G8) by CE was required, coating of capillary with a poly(vinyl alcohol) was mandatory [19]. The coating provided strong reproducibility of

separation for all generations of dendrimers. In contrast to amino-terminated PAMAM dendrimers, a reverse trend of migration for the PAMAM-SAH dendrimers was observed. The migration time was inversely proportional to generations. Due to this reverse trend, the separation of G1–G3 dendrimers was simple. Obviously, the same phenomenon disfavored a separation of higher generations (G4–G5).

Affinity capillary electrophoresis (ACE) can serve for determination of binding constants of vasoactive intestinal peptide to PAMAM dendrimers [21].

Separation of various amines was achieved by dynamic coating CE [51], where the common drawback of capillary zone electrophoresis (CZE) such as the sticking of these solutes with the capillary wall was removed. Dynamic coating CE separated PAMAM dendrimers of seven generations (G0–G6) at pH 7.4, in contrast to CZE. When the polyethyleneimine was used as a dynamic coating agent, the separation at acidic pH was improved.

Synthesis of G5 ethylenediamine-core PAMAM dendrimers with different degrees of acetylation and carboxylation was described [55]. They were investigated as models of the effect of charge and surface modifications of dendrimers on electrophoretic mobility (EM) and molecular distribution. Partial modification of dendrimers led to broader migration peaks than full modification or avoidance of modification. EM was inversely proportional to degree of surface acylation of both PAMAM acetamides and PAMAM succinamides. Due to nonlinear behavior of this phenomenon, the migration activity in CE separations can be hardly explained by charge/mass ratio changes.

The relative mobility of ethylenediamine-core PAMAM succinamic acid dendrimers was driven by molecular weight; hence, for the higher generation of dendrimers the slower migration was observed [59].

Comprehensive characterization of PAMAM dendrimer including the surface functionalization was carried out by Shi et al. [56]. G4 and G5 PAMAM dendrimers with acetamide, hydroxyl, and carboxyl surface group were synthesized, purified by dialysis, and characterized by PAGE, CE, SEC, MALDI-TOF-MS, and NMR.

Coating of capillary column with a carbosilane dendrimer was described [15]. The coating prevented the absorption of Si-O to basic substances such as adenine, adenosine, and 6-furfurylaminopurine. Their total separation was achieved within 20 min. Capillary after double coating provided the best separation effect.

The same laboratory [14] achieved chiral separation by capillary electrophoresis using column coated with carbosilane dendrimers with β-cyclodextrin. The analytes were chlortrimeton, promethazine, and benzedrine. The best resolution of enantiomers was observed for chlortrimeton.

Formation of stable nanoparticles between plasmid DNA (pDNA) and polycationic amphiphilic cyclodextrins served not only for the NA protection but also for separation of nucleic acids by gel electrophoresis [20].

7.3 Mass Spectrometry

For glycopeptides and glycopeptide dendrimers, the most important methods for structure elucidation and determination are mass spectrometries such as FAB-MS, MALDI-TOF-MS, ESI-MS, MS/MS tandem MS, and QqTOF quadrupole-quadrupole time-of-flight [10, 17, 18, 26, 34, 36, 39, 49]. These techniques provide the molecular weight of the product and even they can help in identification and quantification of certain impurities. Furthermore, MS techniques can describe conformation and dynamics space of biomolecules including glycopeptides [28].

ESI-MS methods were used for the determination of K_a values of anion during complexation by glycocluster thioureamethyl calix[4]resorcarenes [44]. It provided fast and quantitative characteristic of the complex formed between a host and a variety of guests.

Ionization of molecules is a hurdle of MS methods [50]. For some compounds, the correct masses were not provided by MALDI-TOF-MS spectra [4, 5, 42]. The results of the MALDI-TOF-MS depended on the molecular mass. With increasing mass (10.8–13.3 kDa), a significant difference was observed between the found (13,357.37 Da) and calculated (13,264.95 Da) masses [4, 5].

Another problem with ionization is observation of "ghost peaks" or "fake defects" [50] by both ESI-MS and MALDI-MS [6, 22, 49]. In the first case, ESI-MS analysis of PPI dendrimers presented a high occurrence of new type of defects, which were confirmed neither by ^{1}H, ^{13}C-NMR spectra nor by MALDI-MS. The second example describes opposite behavior of MALDI versus ESI. ESI-MS of sulfonamide rich dendrimers displayed the spectrum with high sample purity; however, MALDI-MS technique observed signals of defects that seem to be generated during synthesis. Finally, the thermal reactions during ionization within the matrix were responsible of their occurrence instead of synthetic problems. Therefore, mass spectra of dendrimers must be evaluated and interpreted with care, keeping in mind that false-negative and/or false-positive data can be obtained [42, 50].

MALDI-TOF spectra of G4 PEGylated and unPEGylated dendrimers provided mass with approximately 2 % deviations [31]. This relative deviation represents the absolute one around 300 Da.

Standard MALDI-TOF-MS provided masses of ultra-heavy compounds [40], such as immunoglobulin M and G10 PAMAM dendrimer.

Capillary electrophoresis with mass spectrometry was used for glycoscreening in biomedical applications [69].

Screening of molecular recognition and evaluation of self-assembly in supramolecular chemistry of dendrimers are possible by mass spectrometry and were extensively reviewed [48].

Amphiphilic dendrimers can form environment similar to normal and reverse micelles according to properties of the solvent [3]. Sequestration of a guest molecule was achieved by extraction, which was useful for separation of desired compounds. The assemblies mimicking reverse micelles were formed by these compounds with buried carboxylate groups—a hydrophilic and negatively charged interior—capable of sequestration of cations. It was an excellent tool for separation and

preconcentration of cationic peptides from aqueous solutions. The preconcentrated analytes can be directly detected by MALDI-MS. Moreover, the preconcentration amplified compound quantification and led to detection limits down to 500 pM from volumes as small as 250 μL.

During the synthesis of cell-surface epithelial glycopeptide derived from MUC1 by chemical ligation strategy the products and intermediates were characterized using ESI or MALDI-TOF-MS [68]. The increasing molecular weight of glycoconjugate caused an increase of mass spectrum background.

A correlation between degree of conjugation determined by NMR and laser-induced liquid beam ion desorption mass spectrometry (LILBID MS) was more successful than that between NMR and MALDI-TOF-MS [2].

CE with MALDI-TOF-MS was applied for development and validation of a complex dendrimeric contrast agent Gadomer [67]. CE provided separation of Gadomer 24 from related dendrimers and impurities of lower molecular weight. This served for the assurance and control of the quality of complex dendrimeric drug candidate Gadomer. MALDI-TOF-MS data were confirmed by measurements of complementary CE-ESI-TOF-MS [66]. Furthermore, high-resolution Fourier transform cyclotron resonance-MS (FTICR-MS) with/without CE led to mass spectra with the highest mass accuracy and resolution of several impurities presented in low concentrations in various Gadomer batches.

7.4 Miscellaneous Selected Examples

Combination of UV–VIS and EPR spectroscopies was used for investigation of metal–dendrimer interactions [2]. Metal binding of G3 to G5-PPI glycodendrimers containing either a dense maltose or maltotriose was generation dependent. Moreover, based on EPR analysis, internal and external Cu(II) coordination with axial and rhombic symmetry of the generated complexes was observed. External coordination of Cu(II) was facilitated by sugar groups and water molecules.

Electrochemical biosensor for glycated hemoglobin (HbA_{1c}) was developed using dendrimer and boronic acid as modifiers of electrode surface [60]. The detection of glycated hemoglobin was achieved in absence of expensive fluorescence probes and proteins such as anti-HbA_{1c} and haptoglobin. The boronic acid-modified electrode can function as a biosensor for clinical diagnostics of glycoproteins such as HbA_{1c}.

Photoinduced electron transfer from Ru(II) complexes to a quencher was used for design of fluorescence probe for lectins [32]. The sensor contained $Ru(bipy)_3$ core and a derivative of bipyridyl quencher with boronic acids. Lectin binding led to dissociation of carbohydrate boronic acid complex and restoration of natural fluorescence of $Ru(bipy)_3$ system.

Surface plasmon resonance (SPR) is suitable technique for competitive binding assays for recognition of lectin glycopolymer interactions [45]. SPR easily revealed

the availability and bioactivity of Man-modified polymers on the surface during binding of Con A solution.

SPR served for investigation of affinities of mannosylated pentaerythritol core based dendrimers to *E. coli* FimH [64]. Compounds with subnanomolar affinities were described.

Back-scattering interferometry (BSI) is a label-free technique for quantification of carbohydrate–lectin binding [35]. Interactions between saccharides and lectins were rapidly determined. Size-dependent adsorption of sugar-functionalized PAMAM dendrimers was observed and correlated with the expected density of lectins on the surface. The BSI is as sensitive as SPR and quartz crystal microbalance techniques; sometimes it is even better.

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